

2015

Salt Tolerance of *Lactococcus lactis* R-604 as Influenced by Exposure to Various Stress Conditions

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SALT TOLERANCE OF *LACTOCOCCUS LACTIS* R-604 AS INFLUENCED
BY EXPOSURE TO VARIOUS STRESS CONDITIONS

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

the School of Animal Science

by

Ernesto Emilio Gonzalez Duran
B.S., Escuela Agrícola Panamericana Zamorano, 2012
December 2015

ACKNOWLEDGEMENTS

The author would like to acknowledge the support of his family and friends through all of his academic formation. The author would also like to express his respects, admiration and thankfulness to Dr. Kayanush Aryana for all his time, patience, mentorship, support and guidance throughout his studies at Louisiana State University. Recognition are also extended to his committee members Dr. Charles Boeneke and Dr. Marlene Janes for their advice and time. He would also like to thank the faculty and staff from the School of Animal Science and special thanks to the LSU AgCenter and Dr. Bill Richardson for giving him the opportunity of pursuing higher education and research in such a prestigious university.

The author would also like to acknowledge the contribution of other people in this research specially Dr. Douglas Olson for his technical support and scientific advice. The author is especially grateful for the help provided by all his partners in the Dairy Science Microbiology Laboratory, Dr. Marvin Moncada, Ingrid Osorio, Luis Vargas and Behanis Mena. The author wish to acknowledge his friends Ana Ocampo, Katherine Rubio, Jacob Bella, Jose Brandao, Cristhiam Gurdian, Dr. Gabriela Crespo, Dr. Luis Espinoza for their unconditional support and love. Special thanks to Dr. David Blouin and PhD. Candidate Kenneth Carabante for their advice regarding experimental design and statistical analysis. The author would like to express his gratitude to all members of Zamorano Agricultural Society at LSU for their friendship and support during his stay in Baton Rouge.

None of this work would have been done without each of this laudable contributors.

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ABSTRACT

Lactococcus lactis is a dairy culture bacterium widely used in dairy products that contain salt (NaCl) such as cheese and salted butter. Osmotic conditions generally hinder the growth of both pathogen and desirable bacteria. It has been observed that many stress-induced proteins are produced after exposure to an environmental stress protecting the cell against other stresses since the first exposure starts the defense mechanisms of the cells creating an effect of cross-protection. If salt tolerance is enhanced in desirable bacteria they would survive better in salty environments. The objective of this study was to evaluate the salt tolerance of *Lactococcus lactis* R-604 after exposure of various stress conditions. The culture was subjected to 10% v/v ethanol for 30 minutes, 15 mM of hydrogen peroxide for 30 minutes, mild heat at 52°C for 30 minutes and UV light (245 nm) for 5 minutes. The culture was also subjected to starvation (no lactose in M17 broth) for 24 hours or prior osmotic adaption (3% w/v NaCl in M17 broth) for 24 h aerobically at 30°C. A control was run without any stress under the same conditions of each experiment. An initial concentration of 7 log CFU's/mL was used for all treatments. Growth was determined under 5 concentrations of NaCl (0, 1, 3, 5 and 7% w/v). Plating was done every 24 h for 5 days in M17 agar with 0.5% w/v of lactose and incubated aerobically at 30°C for 48 hours. Salt tolerance was enhanced after mild heat or ethanol exposure at 5% w/v NaCl on days 3, 4 and 5. Salt tolerance was also enhance after hydrogen peroxide stress at 5% w/v NaCl on days 4 and 5 and after 24 hours of lactose starvation at 3% w/v on day 3. *L. lactis* R-604 was not negatively affected by any of the stress conditions applied at salt concentrations of 0, 1 and 3% w/v NaCl. The culture was able to grow in 3% NaCl or no lactose after 24 hours and these stress conditions did not affect its salt tolerance. Growth was maintained at 7% w/v NaCl regardless the stress

conditions, however combination of ethanol or hydrogen peroxide at 7% w/v NaCl reduced growth of *L. lactis* R-604.

CHAPTER 1: INTRODUCTION

1.1 *Lactococcus lactis*

Lactococcus lactis is a homo-fermentative Gram positive, ovoid shape, non- motile and non-spore forming bacteria usually within 0.5 and 1.5 μm size (Robinson, 2000). Its ability to produce lactic acid from lactose and other sugars makes it one the most widely used culture in dairy and meat fermented products (Al-Zoreky and Sandine, 1991). *L. lactis* is also widely used for the health benefits that produce once it colonizes the intestinal tract of humans and works as a probiotic (Heller, 2001). It can be used as a vehicle to deliver proteases such as Elafin against inflammatory bowel diseases (Bermúdez-Humarán et al., 2013) or to release bacteriocins against *E. coli* or *Listeria innocua* (Todorov et al., 2007).

Lactococcus lactis has been genetically divided into two subspecies and one biovar: *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis* and *L. lactis* subsp. *lactis* biovar *diacetyllactis* (Samarzija et al., 2001, Itoi et al., 2008, Lahtinen, 2012). Within the three subspecies *L. lactis* subsp. *lactis* is considered the most widely used by its ability to grow under different environmental conditions like alkaline pH, mild salt concentrations (4%) and low heat (40° C) treatment (Kim et al., 1999).

Cheese is one of the most important dairy products and it is usually made out of pasteurized milk which is acidified using dairy cultures or organic acids (El-Salam et al., 1999). Chymosin is added and this produces the coagulation of milk protein (casein), then is salted and it can be ripened from a few days to several months to create different flavor and aroma profiles (Yvon et

al., 1997). During the ripening period it also produces intracellular enzymes and other metabolites that develop desirable characteristics such as texture, aroma and flavors (Carolina and Nestor, 2013). Usually a blend of different species of dairy cultures including *Lactococcus lactis* are sold for commercial and industrial use for dairy products manufacture (Robinson, 2000).

Lactococcus lactis is used in the manufacturing of many types of cheeses, such as: Cheddar, Brie, Camembert, Parmesan, Colby and others. Most of the commercial cheese contains less than 2% of salt (w/w) but there are some pickled cheeses that may contain between 3 and 15% of salt (w/w) like Domiati (El-Baradei et al., 2007), Nabulsi (Al-Dabbas et al., 2014), Akawi, Feta and Blue cheeses (Boutibonnes et al., 1991). Most of the cheese commercially available in the United States (cheddar and mozzarella being the most common) have between 1.5 and 2% of salt content and they accurately declare their sodium content (Agarwal et al., 2011). Cheese is one of most important sources of sodium in regular diets but there is an increasing trend in sodium reduction in foods since high intake of sodium has been associated with development of chronic disease such as high blood pressure, heart diseases, strokes and kidney failure (Appel et al., 2011). Nevertheless, in some gourmet cheeses reduction of salt content may be difficult because it contributes to the development of desirable characteristics and flavor profile (Hashem et al., 2014).

1.2 Salt tolerance

In many dairy products salt is used as a preserving agent, as a proteolysis enhancer and it is also used because it imparts a desirable flavor. Salt can also affect the quality and flavor development in the ripening process since it inhibits microbial activity of both spoilage and beneficial bacteria such as lactic acid bacteria and probiotics. Smith et al. (2010) while studying the molecular

mechanisms of stress resistance in *Lactococcus lactis* stated that at salt concentrations of 3 to 5% w/v NaCl, the culture produces more lactic acid but its growth is inhibited at 6% w/v NaCl. Concentrations above 6% can drastically reduce the growth of *L. lactis* by 80% and reduce by 50% the acid production (Tripathi et al., 2003). Uguen et al. (1999) while studying the influence of the presence of osmoprotectant (i.e. glycine betaine) on the growth of *L. lactis* ADRIA 85LO30 found that a salt concentration of 0.5 M (approximately 3% w/v NaCl and 3.6% w/v KCl) in chemically defined medium (CDM) the growth rate was reduced by 70%.

Higher concentration of salt in a medium will cause an osmotic stress in bacteria, since it creates an imbalance of ions (K^+ , Na^+ and Cl^-) concentration in the cytoplasm of the cell and the environment. Dehydration of the cell can be produced by the release of water through the permeable cell membrane to maintain homeostasis (Obis et al., 1999). Also this permeability of the membrane allows salt ions to move into the cytoplasm affecting cell functions such as gene expression, protein synthesis and water retention which is necessary for cell nutrition and stability (Rallu et al., 1996).

Some bacteria protect themselves by absorbing or producing other type of solids to counteract the adverse effect of salt ions in the environment. These solids are known as “compatible solutes”. These compatible solutes are highly water soluble and do not affect cell function even if they are present at very high concentrations and can be produced by modification of the cell’s enzyme activity or at a transcriptional level (Yousef and Courtney, 2003). Compatible solutes can be carbohydrates or amino acids such as proline, glycine-betaine and glutamate (van de Guchte et al., 2002). When bacteria increase the solute content of the cytoplasm, it increases the internal osmotic pressure and keeps the cell turgor protecting the cell from dehydration (Beales,

2004). Some strains of *Vibrio* have the genes to produce compatible solutes such as glycine betaine from choline (Kapfhammer et al., 2005).

L. lactis has the ability to produce and retain betaine, this is stimulated by an osmolality threshold (O'Callaghan and Condon, 2000). Betaine uptake in *L. lactis* is regulated in both genetic and biochemical levels and its presence in the growth media increase *L. lactis* salt tolerance since it protects intracellular macromolecules from denaturation and the cell itself from dehydration (Obis et al., 1999).

Resistance to salt can be achieved by adding betaine solutes in incubation medium (O'Callaghan and Condon, 2000) or by the addition of genes to the bacteria genome which increase betaine uptake such as BetL (Sheehan et al., 2006). Beside the betaine uptake *L. lactis* shows another mechanism to survive hyperosmotic conditions that is the production of membrane associated proteins like HtrA (Foucaud-Scheunemann and Poquet, 2003) and FtsH (Nilsson et al., 1994). Foucaud-Scheunemann and Poquet (2003) found that HtrA heat stress protein was produced under various stress conditions such as medium salt concentration (4.5% NaCl), ethanol (up to 10%) and low heat shock (less than 55° C). This showed that bacteria may have similar stress responses for different stress conditions. However, there are also specific responses for a determined stress, for instance there is one salt stress induced protein denoted as ssp21, which is produced when *L. lactis* cells are grown in 2.5% of NaCl concentrations (Kilstrup et al., 1997).

Fatty acid composition of cellular membrane can be modified to protect the cell from hyperosmotic conditions (van de Guchte et al., 2002). An increase in the ratio of trans to cis fatty acid has been also observed in osmotically stressed cells (Cronan, 2002). An increase in cyclopropane fatty acid (Δ C19:0) and lower levels of oleic acid (C18:1) which has been

observed in *L. lactis* has been considered to enhance betaine transport and regulate permeability (Smith et al., 2010).

1.3 Ethanol stress

Alcohol is a metabolite of many fermentative microorganisms and is produced out of simple sugars. When the alcohol levels in the environment reach toxic levels, bacterial cells start to die off. Although lactic acid bacteria is well known to survive in high concentrations of ethanol (from 14 to 20% v/v), the resistance mechanisms are not well understood and survival rate can be reduced in about 90% with only 25% v/v of ethanol in growing broth (Kubota et al., 2008, Liu and Qureshi, 2009).

Ethanol is known to cause change in the composition and function within bacterial cells, also inhibit cell division, decrease viability and sugar transport system and affect organelles (mitochondria and vacuoles) functions. Gram-negative bacteria are especially vulnerable to ethanol exposure while Gram-positive bacteria such as lactic acid bacteria are known to survive and even grow in high ethanol environments, up to 6% without affecting its growing capacity (Liu and Qureshi, 2009). Ethanol at 4% v/v pre-exposure for 30 minutes confer heat resistance (50°C for 25 minutes) to other Gram-positive bacteria like *Bacillus cereus* by stimulating the production of heat stress proteins such as GroEL, DnaK and FtsH (Periago et al., 2002). A compilation of studies presented by Taylor et al. (2008) showed that bacteria can respond physiologically to ethanol presence by increasing unsaturated fatty acid in the cell membrane and increasing production of heat stress proteins. Gonzalez et al. (2003) reported that *E. coli* increases the glycine metabolism and the betaine production after ethanol exposure (4% v/v), if this response applies to *L. lactis* salt tolerance might be enhanced after ethanol stress.

L. lactis exposed to ethanol (10% v/v) for 30 minutes among other stresses (heat at 39°C, and 4.5% w/v NaCl), increased htrA transcription which is a membrane associated protein that is essential for the cell survival under heat and oxidative stress (Foucaud-Scheunemann and Poquet, 2003) but the influence of ethanol exposure on the salt tolerance of *L. lactis* was not evaluated. Exposure of either 10 or 30 minutes to ethanol at 8% v/v in MRS medium enhances heat tolerance of *Lactobacillus plantarum* by changing the cell membrane fatty acid composition and also triggering genes expression for the transcription of protein associated membranes such as htrA, groEL-groES and dnaK-dnaJ which are also produced under heat stress (van Bokhorst-van de Veen et al., 2011).

1.4 Mild heat treatment

Heat is one of the most damaging features that can stress bacteria, and it is widely used to kill undesirable microorganisms since it denatures cell macromolecules (Smith et al., 2010). *Lactococcus lactis* cells start to be negatively affected at temperatures of 48° C and above (Boutibonnes et al., 1991) and their optimal growth temperature is 30° C (Smith et al., 2010). Heat affects bacteria by damaging fatty acids in the cell membrane or ribosomes (Corcoran et al., 2008). Heat also denatures bacterial proteins and subsequently affects their functions (van de Guchte et al., 2002). Some bacteria after heat stress also increase the ratio of trans to cis fatty acid in the cell membrane, this change may reduce permeability of the cell (Cronan, 2002).

When most bacterial cells are under heat stress, they encode several proteins that act as chaperones to repair or destroy damaged structures in the cell (Yousef and Courtney, 2003). Some examples of heat induced proteins are RecA, groEL, grpE, dnaK and hflB, which are involved in DNA repair and other protein synthesis (van de Guchte et al., 2002). These proteins are also associated with increasing the resistance to oxidative stress, acidity and cold temperature

shock (Smith et al., 2010). Exposure to heat above the optimal growth temperature of microorganisms can activate resistance mechanisms which may increase their tolerance to other lethal stresses (Boutibonnes et al., 1991). *Lactococcus lactis*, showed an increase in its cryotolerance by having a 48% survival rate after a mild heat treatment (42°C for 25 minutes) compared to 20% survival of the control without low heat treatment (Broadbent and Lin, 1999). It has been shown that *L. lactis* under osmotic stress (2.5% w/v NaCl) was induced to produce heat stress proteins such as DnaK, GroEL and GroES, this overlap in protein production can enhance resistance to heat after a short osmotic shock (Kilstrup et al., 1997). However there are no studies that evaluate the influence of mild heat on the growth of *L. lactis* at different salt concentrations.

1.5 Oxidative stress

Oxidative stress can be defined as the cells response to an excessive accumulation of reactive oxygen compounds in the environment (Rochat et al., 2005). Oxidative agents can cause damage in the cell when molecules of O_2^- and OH^- radicals get attached to organic compounds (proteins, lipids and nucleic acids) inside the cell, which may cause cell death. Some proposed mechanisms of oxidative stress protection are regulation of internal cell oxidation molecules (enzymes such as recombinase RecA to protect DNA), minerals (iron, manganese, copper or zinc) and other oxidative regulators like phosphates (Smith et al., 2010).

At a molecular level oxygen radicals can react with proteins and nucleic acids. It can also react with some cations such as Fe^{2+} and Cu^+ . Oxygen can also attack the DNA chains and oxidize the lipid membrane affecting its permeability. Oxygen stress affect the metabolic function of *L. lactis* mostly its lactic acid production capability, which tend to be reduced. Other metabolites like CO_2 , ethanol, acetate, acetoin and diacetyl tend to increase when bacteria are exposed to

oxygen reagents (Miyoshi et al., 2003). Bacteria is very sensitive to hydrogen peroxide, only 15 mM is considered a lethal challenge for *L. lactis* (Hartke et al., 1995) and about the same amount (10 mg of H₂O₂/L) are lethal for *Staphylococcus aureus* (Dahiya and Speck, 1968). Other studies used a range of 0.5 to 1.15 mM of hydrogen peroxide to test for oxidative tolerance (Hartke et al., 1995, O'Sullivan and Condon, 1997, Foucaud-Scheunemann and Poquet, 2003, Miyoshi et al., 2003, Rochat et al., 2005). But there are no studies that have evaluated the influence of oxidative stress on the salt tolerance of *L. lactis*.

Lactococcus lactis and other lactic acid bacteria are very vulnerable to oxidative stress since they lack catalase activity to reduce hydrogen peroxide (H₂O₂) into two molecules of water (H₂O) and Oxygen (O₂) (Rochat et al., 2005). Some lactic acid bacteria have the ability to produce easy reduction molecules to protect vital compounds from the oxidative agents in the environment, this molecules are oxidized instead of the bacteria vital compounds (Miyoshi et al., 2003). Another defense mechanism of *L. lactis* against oxidative stress is changing in the fatty acid composition of the cell membrane which increases the oxygen consumption of the fatty acid desaturase system, which eliminates oxygen radicals (Miyoshi et al., 2003).

1.6 Irradiation stress (UV light)

Ultraviolet light (UV) in the range of 200 to 280 nm has been considered an alternative in food processing to inactivate pathogens and spoiler organisms mostly in beverages like water, juices, apple cider, milk and even in some produce like lettuce (Lu et al., 2011). UV light treatment of milk has shown potential to inactivate pathogens such as *Staphylococcus aureus* (Krishnamurthy et al., 2007), *Listeria monocytogenes* (Matak et al., 2005) and *Mycobacterium avium* (Altic et al., 2007). The main disadvantage of UV light treatment is that it is only effective on surfaces and milk composition (mainly proteins and fats) reduces penetration. Adequate UV wave length,

irradiance, equipment design, and proper flow can achieve more than 6 Log reduction of some of the milk related bacteria (Lu et al., 2011)

Lactococcus lactis strains after 30 min under UV light (254 nm) and 100 KJ/m² can reduce viability in almost 90% and also produce stress induced proteins like GroEL and GroES that are also produced by heat shock and seem to be related to help protect bacteria against other environmental stresses such as acid challenge (Hartke et al., 1997). Exposure of *L. lactis* to UV light has shown an increase in resistance to lethal challenges of other stresses such as acid, ethanol, heat and hydrogen peroxide, since the pre-exposure to UV light encourages the production of a protein (U1) which protect the DNA of the cell from other stresses (Hartke et al., 1995).

Like oxidative stress, UV light attacks the cell's DNA and it is used a mutagenic tool in several microorganisms (Miller, 1985, Pfeifer et al., 2005). Gram negative bacteria have more repair DNA mechanisms and they are more resistant to radiation than gram positive (like *Lactococcus lactis*). For gram negative bacteria four protection mechanisms have been proposed: RecA (heat induced protein) synthesis to protect DNA, chemical modification of the cell membrane, permeability changes in the cell membrane, and reduction in cell division which saves DNA for other uses. Some of these mechanisms may be used by *L. lactis* when it is exposed to UV light (Duwat et al., 1997).

1.7 Adaptation

Adaptation is an evolutionary process whereby an organism becomes better able to live under certain conditions (Dobzhansky, 1968). Pre-exposure to sub-lethal levels of stress turns the cell more resistance to lethal levels of the same stress. For instance, *L. lactis* strains cultivated at pH

5 grow better in higher acidic conditions. Acid tolerance response is one of the most studied adaptation treatments in lactic acid bacteria (Rallu et al., 1996, Smith et al., 2010).

Adaptation to 2.5 % w/v NaCl concentrations seems to help *L. lactis* to resist lethal concentrations of 4% w/v NaCl and the long exposure to mild salt concentrations (2.5% w/v NaCl) improves the production of heat and salt tolerance proteins like SSP21 (Kilstrup et al., 1997). Adaptation to heat stress has also been found in *L. lactis*, exposure to non-lethal temperatures for short times improves survival rate at higher (normally lethal) temperatures (Rallu et al., 1996). Adaptation can also be induced for cold temperatures, *Lactobacillus* strains increase freezing survival in about 2 log after a 15°C treatment for 2 hours (De Angelis and Gobetti, 2004). A study using *L. lactis* ssp. *lactis* and *cremoris* showed that pre-exposure to mild acid conditions, low heat shock and low bile salt concentration increase the viability of the culture to lethal levels of the same stress (Kim et al., 1999).

Many strains of *Lactococcus* (Hartke et al., 1996), *Lactobacillus* (Lorca et al., 2002), and *Propionibacterium* have been found to be more resistant to lethal challenges of acid after a pre-exposure to mild levels of the same stress (adaptation). In the case of *Propionibacterium* it was found to be more resistant to high acidic conditions (pH 2.0 for 30 minutes) after exposure to a pH of 5.0 for 60 minutes, enhancing their survival rate from 43% (no previous exposure to acid) to 100% after adaptation (Jan et al., 2001).

Adaptation of *Bifidobacterium adolescentis* was observed after exposure to 0.1% w/v bile for 30 min, since it was able to survive at higher levels of bile (0.4 and 0.5 % w/v) of about 300 fold (Schmidt and Zink, 2000). Pre-adapted *Lactobacillus acidophilus* cells showed higher survival rate (about 2 Log) to lethal levels of heat (60° C) and bile (0.5% w/v) after exposing them to sub-lethal levels of the same stress (53° C and 0.05% w/v respectively). For lethal levels of NaCl

(18% w/v), the increase in survival rate was only from 46% to 74% after exposure to the sub-lethal level (2% w/v NaCl)(Kim et al., 2001).

L. lactis cells collected after a severe stress challenge including low acid (pH of 2.8), bile salt (0.1% of bile salt), heat (49° C) and hydrogen peroxide (3 mM of H₂O₂), were harvested and grown again in the same conditions and they showed a higher survival rate than the first time. This meant that the original survivor cells were better adapted to the lethal stress conditions. Usually adaptation is achieved when the bacterial cells are exposed to mild stress for a period of time, giving the chance to synthesize proteins that increase the survival rate (Kim et al., 2002).

1.8 Starvation

Starvation is subjecting bacterial cells into a medium with low or no nutrients, or where their availability is reduced due to modifications in environment like extreme pH or low water activity (van de Guchte et al., 2002). Lactic acid bacteria react under starvation with modifications in their cell morphology, usually tending to shrink, slowing the rate of cell division, modification in the cell membrane fatty acid composition and decreased protein synthesis (Hartke et al., 1994).

Carbohydrate starvation usually leads to a quick exponential growth, but when the cell reserves are gone they become a viable dormant culture (Sanders et al., 1999). Resistance of this stress is determined by lipid membrane integrity, cytoplasmic enzymes activity and CcpA gene regulation (Smith et al., 2010) After starvation, Gram- positive bacterial expression of sigma factor (protein necessary for RNA synthesis denoted by σ^B) is increased, this factor is also involved in the control of catalase synthesis (katE) and the transport of osmoprotectants such as proline (opuE) (Pichereau et al., 2000).

Starvation induced synthesis of stress proteins are produced under harsh environmental conditions such as ethanol, heat, oxidative and osmotic stress (Jenkins et al., 1990, Spence et al., 1990, Jouper-Jaan et al., 1992, Hartke et al., 1994). The overlap in stress induced proteins can increase the cell's resistance to other stresses but evidence is not conclusive (Giard et al., 1996). *Enterococcus faecalis* developed resistance against heat (62°C), hydrogen peroxide (20 mM), acid (pH 3.7) and ethanol (17% ethanol) after a glucose starvation period (Giard et al., 1996). Glucose starved cultures of *Lactococcus lactis* subsp. *lactis* IL1403 were found to develop better survival rate to ethanol stress (20%), oxidative stress (15 mM of hydrogen peroxide), low heat stress (52° C) and up to 0.35 M (approximately 2% w/v) of sodium chloride (Hartke et al., 1994). There is no research involving the influence of lactose starvation on the growth of *L. lactis* at various salt concentrations.

1.9 Cross protection

It has been observed that previous exposure of *L. lactis* to low intensity stress conditions encourage resistance to the same stress at lethal levels, because this first exposure starts the defense mechanisms of the cell (Hartke et al., 1994, Kim et al., 1999, Sanders et al., 1999, Tripathi et al., 2003). There have also been observations where exposure to an environmental stress can develop resistance to different types of stress since there are many stress-induced proteins and low molecular weight compounds that are produced under more than one stress condition creating an effect of cross protection (Pichereau et al., 2000).

The overlapping levels of control, when the cell synthesizes stress induced proteins, were believed to be specific for each stress, but recent studies have shown that heat stress proteins (RecA, GroEL and GroES) are also produced in oxidative stress, starvation, osmotic and UV

light exposure (Hartke et al., 1995, Lewis et al., 1995, Kilstrup et al., 1997, Sanders et al., 1999, van de Guchte et al., 2002). Exposure of *L. lactis* at pH 5.0 for 2 hours increased its survival rate by 2 logarithms under lethal levels of other stresses including heat shock (42° C), NaCl (20 w/v), H₂O₂ (1.15 mM) and ethanol (15% v/v) (O'Sullivan and Condon, 1997). In another study, *L. lactis* also showed an effect of cross protection after UV light exposure (60 and 100 J/m²) against heat shock (52° C), acid conditions (pH 4.0) and ethanol (20% v/v) by increasing its survival in about 2 logarithms (Hartke et al., 1995). Pre-exposure to sub-lethal levels of bile (0.05% w/v) showed an increase in survival up to 1 log to lethal levels of bile (0.5 % w/v), heat shock (60° C), but not for NaCl (18% w/v) (Kim et al., 2001). *L. lactis* exposed to heat treatment (40°C) and nisin in medium demonstrated an increase in tolerance of osmotic stress (4% NaCl), ethanol stress (5%) and mild acidic conditions (pH 5.47) (Abdullah Al et al., 2010).

1.10 Justification

There are more than 10 billion pounds of cheese produced in the United States every year (Paraman et al., 2013) and cheese is one the main sources of dietary sodium in regular diets (McGuire, 2011). Salt content in most commercial cheeses can range from 1.5 to 3% w/v (Hashem et al., 2014). Some Mediterranean cheeses like Domiati and Feta can have between 4 and 6% w/v salt, which is necessary to preserve the cheese and contribute to its flavor and also as a source of dietary sodium (Guinee, 2004). Besides the fact that there is an increasing effort to reduce sodium in foods, salt is an important ingredient because it is used to enhance flavor and to control bacteria (coliforms and pathogens) and undesirable mold growth.

Sodium is an essential nutrient and constitutes more than 90% of the cations in human's blood stream playing an important role along with potassium and chloride in the osmotic pressure regulation, acid-base metabolism and absorption of nutrients (Escott-Stump et al., 2015). Sodium

is also involved in maintaining blood pressure and muscle movement (DeBruyne et al., 2015). The Dietary Guidelines Advisory Committee (2015) for the 2015 USDA dietary guidelines increased the tolerable upper intake level from 2300 to 2400 mg of sodium per day (based on a 2000 kCal diet) even though high sodium consumption has been associated with high blood pressure and cardiovascular diseases (Cook et al., 2007).

Development of techniques to make dairy cultures and probiotics stronger is necessary since during food processing, cultures are exposed to adverse conditions such as high heat, high acid and high salt concentrations. Sales of probiotic products was estimated to be more than \$30 billion in 2015 (Statista 2015). If salt tolerance is enhanced in desirable bacteria they would survive better in salty environments. This could create an opportunity to produce new probiotic/functional products that traditionally have high salt content such as Mediterranean cheeses, cured meats (up to 8% w/v NaCl), canned vegetables (up to 5% w/v NaCl), soups (up to 20% w/v NaCl) or sauces (up to 5% w/v NaCl) (Mhurchu et al., 2011).

There are some resistance mechanisms that have been characterized but there is no research involving the influence of environmental stress exposure on the salt tolerance of the cheese bacterium *Lactococcus lactis* R-604. Taking advantage of the capacity of *Lactococcus lactis* to adapt from stressful environment and in order to test a proposed cross-protection mechanism, this study was conducted with six different environmental stress condition prior to the exposure to five levels of NaCl to determine if its salt tolerance can be enhanced.

The hypothesis was whether the subjection of prior mild stress conditions would enhance salt tolerance of *Lactococcus lactis* R-604. The objective was to study the effect of six different stress exposures (ethanol, mild heat, hydrogen peroxide, UV light, adaptation and lactose

deprivation) on the growth of *Lactococcus lactis* R-604 in five levels of NaCl (0, 1, 3, 5 and 7% w/v).

CHAPTER 2: MATERIALS AND METHODS

2.1 Experimental design

The treatments consisted of ethanol stress (30 minutes incubation in M17 broth at 30°C broth with 10% v/v ethanol), oxidative stress (30 minutes incubation in M17 broth at 30°C with 5mM of H₂O₂), mild heat (50°C for 25 minutes), UV light exposure (254 nm for 5 minutes), a control for these treatments which was conducted without any stress. The second part of the experiment involved a growth period of 24 hours using osmotic adaptation (24 hours incubation in M17 broth at 30°C with 3% w/v of NaCl) and lactose deprivation (24 hours incubation in M17 at 30°C with no lactose), a different control was grown for 24 hours in M17 broth as. After each treatment the culture was subjected to five NaCl concentrations (0, 1, 3, 5 and 7% w/v). Bacterial counts were determined every 24 hours for 5 consecutive days. The experiments were conducted in duplicate and repeated three times. Data were analyzed as a complete randomized split plot design with repeated measures over time.

2.2 Preparation of media

2.2.1 Peptone water

A solution of 0.1% w/v of peptone water was prepared according to manufacturer specifications dissolving 1g of peptone powder (Bacto™ Peptone, Difco, Dickinson and Co., Sparks, MD) in 1L of distilled water. Peptone solution (99 mL) was poured into clean dilution bottles and then sterilized at 121°C for 15 minutes in an autoclave (AMSCO Scientific, Erie, PA).

2.1.2 Preparation of broth

M17 broth was used for all samples and it was prepared according to manufacturer specifications as follow: 37.25 grams of M17 broth powder (Fisher Scientific, Fair Lawn, NJ) was diluted in 1L of distilled water, then 94 mL of the solution was poured into different bottles and it was sterilized at 121°C for 15 min. Also a 10% w/v lactose solution (Fisher Scientific, Fair Lawn, NJ) was prepared separately and sterilized under the same conditions. After autoclaving, 5 mL of the lactose solution was aseptically added into the sterile M17 bottles. Then the bottles were tempered at 30°C in an aerobic incubator.

2.1.3 Agar preparation

M17 agar was used for all samples. It was prepared according to manufacturer specifications as follow: 37.25 grams of M17 broth powder (Fisher Scientific, Fair Lawn, NJ) and 12 g of pure agar powder (Fisher Scientific, Fair Lawn, NJ) were diluted in 1 L of distilled water, heating and mixing them in hot plate with a magnetic stirrer until the solution boiled. Then it was sterilized at 121°C for 15 min. Lactose solution (10% w/v) was prepared separately and sterilized under the same conditions. After autoclaving, 50mL of the lactose solution was aseptically added into the sterile M17 agar and it was kept in a water bath at 60°C until used.

2.2 Treatments protocols for Group 1

2.2.1 Control 1

Pure culture of *Lactococcus lactis* DVS culture R-604 (CHR HANSEN, Milwaukee, WI) was thawed and inoculated at approximately 10^9 CFU/mL in M17 broth supplemented with 0.5% w/v

lactose. After that, a sample of 1mL of this dilution was transferred to five different bottles containing sterile M17 broth with NaCl (Fisher Scientific, Fair Lawn, NJ) at 0, 1, 3, 5, and 7% (w/v) and immediately plated. Bacterial counts (CFU/ mL) were determined by plate counting in M17 agar with 0.5% w/v lactose (Kim et al., 1999, O'Callaghan and Condon, 2000). This was the initial time point for the experiment and hence it was called day 0. The inoculated M17 broths with the five salt concentrations were incubated aerobically at 30° C (Kim et al., 2002) for 5 days. Samples were drawn every 24 hours.

2.2.2 Ethanol treatment

This experiment was conducted according to Tian et al. (2012) with slight modifications. Pure culture of *L. lactis* R-604 CHR HANSEN was thawed and inoculated at approximately 10^9 CFU/mL in M17 broth supplemented with 0.5% w/v lactose and containing 10% (v/v) of ethanol (200° proof). Samples were incubated aerobically for 30 minutes at 30°C. After this, a sample of 1 mL of this dilution was transferred to five different bottles containing sterile M17 broth with Sodium Chloride (NaCl) at 0, 1, 3, 5, and 7% (w/v) and immediately plated. Bacterial counts (CFU/ mL) were determined by plate counting in M17 agar with 0.5% w/v lactose. The inoculated M17 broths with the five salt concentrations were incubated aerobically at 30° C for 5 days. Samples were drawn every 24 hours.

2.2.3 Hydrogen peroxide treatment

This experiment was conducted according to Hartke et al. (1995) with slight modifications. Pure culture of *L. lactis* R-604 CHR HANSEN was thawed and inoculated at approximately 10^9 CFU/mL in M17 broth supplemented with 0.5% lactose and containing 15 mM of hydrogen peroxide (Pharmco-Aaper, Brookfield, CT). Samples were incubated aerobically for 30 minutes

at 30°C. After this, 1mL of this dilution was transferred to five different bottles containing sterile M17 broth with NaCl at 0, 1, 3, 5, and 7% (w/v) and immediately plated. Bacterial counts (CFU/mL) were determined by plate counting in M17 agar with 0.5% w/v lactose. The inoculated M17 broths with the five salt concentrations were incubated aerobically at 30° C for 5 days. Samples were drawn every 24 hours.

2.2.4 Mild heat treatment

Pure culture of *L. lactis* R-604 CHR HANSEN was thawed and inoculated at approximately 10^9 CFU/mL in M17 broth supplemented with 0.5% lactose. The inoculated broth was heated at 52°C for 30 minutes in a water bath and rapidly chilled in ice cold water (Boutibonnes et al., 1991, Hartke et al., 1994, Hartke et al., 1997, Sanders et al., 1999, Ventura et al., 2005). Then a sample 1 mL of this dilution was transferred to five different bottles containing sterile M17 broth with NaCl at 0, 1, 3, 5, and 7% (w/v) and immediately plated. Bacterial counts (CFU/ mL) were determined by plate counting in M17 agar with 0.5% w/v lactose. The inoculated M17 broths with the five salt concentrations were incubated aerobically at 30° C for 5 days. Samples were drawn every 24 hours.

2.2.5 UV light exposure treatment

This experiment was conducted according to Hartke et al. (1995) and Duwat et al. (1997) with some modifications. A 10 mL sample of pure culture of *L. lactis* R-604 CHR HANSEN was aseptically poured into a sterile petri dish and left uncovered 65 cm away from the UV source (Sylvania Germicidal 30W fluorescent tube) at 254 nm for 5 minutes in laminar flow cabinet (Purifier Class II, Labconco Corp. Kansas City, MO), 1mL of the irradiated culture was taken and serially diluted in sterile M17 broth at approximately 10^9 CFU/mL, then a sample of 1mL

from this dilution was transferred to five different bottles containing sterile M17 broth with NaCl at 0, 1, 3, 5, and 7% (w/v) and immediately plated, Bacterial counts (CFU/ mL) were determined by plate counting in M17 agar with 0.5% w/v lactose. The inoculated M17 broths with the five salt concentrations were incubated aerobically at 30° C for 5 days. Samples were drawn every 24 hours.

2.3 Treatments protocols for Group 2

2.3.1 Control 2

Pure culture of *L. lactis* R-604 CHR HANSEN was thawed and inoculated at approximately 10^9 CFU/mL in M17 broth supplemented with 0.5% lactose. Samples were incubated aerobically for 24 hours at 30°C. After this, 1mL of this dilution was transferred to five different bottles containing sterile M17 broth with NaCl at 0, 1, 3, 5, and 7% (w/v) and immediately plated. Bacterial counts (CFU/ mL) were determined by plate counting in M17 agar with 0.5% w/v lactose. The inoculated broths with the five salt concentrations were incubated aerobically at 30° C for 5 days. Samples were taken every 24 hours.

2.3.2 Osmotic adaptation treatment

This experiment was conducted according to Kilstrup et al. (1997), Sanders et al. (1999) and Tripathi et al. (2003) with some modifications. Pure culture of *L. lactis* R-604 CHR HANSEN was thawed and inoculated at approximately 10^9 CFU/mL in M17 broth supplemented with 0.5% lactose and containing 3% (w/v) of NaCl. Samples were incubated aerobically for 24 hours at 30°C. After this, a sample of 1mL of this dilution was transferred to five different bottles containing sterile M17 broth with NaCl at 0, 1, 3, 5, and 7% (w/v) and immediately plated. Bacterial counts (CFU/ mL) were determined by plate counting in M17 agar with 0.5% w/v

lactose. The inoculated M17 broths with the five salt concentrations were incubated aerobically at 30° C for 5 days. Samples were taken every 24 hours (Kilstrup et al., 1997, Sanders et al., 1999, Tripathi et al., 2003).

2.3.3 Starvation treatment-Lactose deprivation

This experiment was conducted according to Kunji et al. (1993), Hartke et al. (1994) and van de Guchte et al. (2002). Pure culture of *L. lactis* R-604 CHR HANSEN was thawed and inoculated at approximately 10⁹ CFU/mL in M17 broth with no lactose. Then it was incubated aerobically for 24 hours at 30°C. After this, a sample of 1mL of this dilution was transferred to five different bottles containing sterile M17 broth with NaCl at 0, 1, 3, 5, and 7% (w/v) and immediately plated. Bacterial counts (CFU/ mL) were determined by plate counting in M17 agar with 0.5% w/v lactose. The inoculated M17 broths with the five salt concentrations were incubated aerobically at 30° C for 5 days. Samples were taken every 24 hours.

2.3.4 Sample plating

Samples were taken from the M17 broth with the different salt concentrations specified above and serially diluted in sterile peptone. A sample of 1mL was taken and aseptically poured in sterile petri dishes. M17 agar supplemented with 0.5% (w/v) lactose was pour plated over the sample. Inoculated plates were incubated aerobically at 30°C for 48 hours and then counted.

2.4 Statistical Analysis

Data were analyzed separately as a complete randomized design split plot with repeated measures over time using Proc Glimix of SAS (version 9.3 SAS Institute Inc., Cary, NC). Stress conditions, salt concentrations and time of exposure were fixed effects, whereas replicates were random effects. Statistical analysis was made separately for each group of experiments.

Significant differences were tested with least square means at $P < 0.05$ for main effects (stress conditions, time and salt concentration) and the interaction effect (stress conditions \times salt concentrations \times time of exposure). Significant differences ($P < 0.05$) between pair comparisons of interest were analyzed using a LS Means.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Influence of various stress conditions on salt tolerance of *Lactococcus lactis* R-604.

Counts of *Lactococcus lactis* R-604 at various salt concentrations over 5 days of salt exposure are presented in Table 1. The salt tolerance of *Lactococcus lactis* R-604 is presented as two separate groups because the time of exposure of the stress conditions were different. Time of exposure for ethanol, hydrogen peroxide, heat shock and UV light were 30 min or less hence were placed in Group 1 to facilitate overall comparison to identify best conditions for enhancing salt tolerance and Group 1 had its own control called Control 1. Time of exposure of osmotic adaptation and lactose deprivation was 24 hours hence was placed in Group 2 to enable comparison to identify best conditions for improving salt tolerance and Group 2 had its own control called Control 2. In Group 1, the three way interaction between stress conditions*salt concentration*days had a significant ($P<0.05$) effect which meant that cell counts of *L. lactis* depended on the stress condition applied at a specific salt concentration over the days of exposure (Table 2). For Group 2, the three way interaction had no significant ($P<0.05$) effect, but the two way interaction (salt concentration*days) showed a significant ($P<0.05$) effect which meant that cell counts were only depended on the salt concentration and days of exposure (Table 2).

3.2 Salt tolerance of *Lactococcus lactis* R-604 with no previous stress (Control 1).

Counts of salt tolerance of Control 1 at various salt concentrations over 5 days are presented in Table 1. On day 0, there were no significant ($P>0.05$) difference in bacterial counts, meaning that all counts of salt levels started at the same 7 log CFU/ mL. On day 1, an increase of 3.5- 4.5

log growth was observed for 0, 1, 3 and 5% w/v NaCl while bacteria subjected to 7% w/v NaCl did not grow. On the second day, a stationary phase was observed at 11 log CFU/ mL for all levels of salt except for 7% w/v NaCl. On the third day, decline phase was observed for 0, 1, 3 and 5% w/v NaCl since they started to reduce counts at rate of approximately 1 log per day, while bacteria subjected to 7% w/v mL started to grow.

Table 1. Least Square Means (LS Means) expressed as log CFU/mL of salt tolerance of *Lactococcus lactis* R-604 influenced by previous exposure to ethanol, hydrogen peroxide, heat shock, and UV light compared to the control (no previous stress).

		Days						
		0	1	2	3	4	5	
Control 1	0	7.17 ^{Da}	11.46 ^{Aa}	11.06 ^{Aa}	10.07 ^{Bcde}	8.9 ^{Cbdef}	8.26 ^{Cbdef}	
	1	7.13 ^{Da}	11.48 ^{Aa}	11.01 ^{Aa}	9.9 ^{Bde}	9.09 ^{BCbde}	8.45 ^{Cbdef}	
	3	7.14 ^{Da}	11.4 ^{Aa}	11.06 ^{Aa}	9.76 ^{Bde}	8.24 ^{Cdefg}	8.04 ^{CDdefg}	
	5	7.10 ^{Da}	10.48 ^{ABab}	11.16 ^{Aa}	10.1 ^{Bbcde}	8.75 ^{Cdefg}	7.02 ^{Dg}	
	7	7.11 ^{Ba}	6.83 ^{Bd}	7.15 ^{Bc}	8.38 ^{Afg}	9.27 ^{Abcd}	8.93 ^{Aabcde}	
Ethanol	0	7.19 ^{Ca}	11.31 ^{Aa}	11.23 ^{Aa}	10.51 ^{Aabcd}	9.32 ^{Bbcd}	9.32 ^{Bab}	
	1	7.19 ^{Da}	11.37 ^{Aa}	11.09 ^{Aa}	10.03 ^{Bde}	8.87 ^{Cbdefg}	8.86 ^{Cabcde}	
	3	7.15 ^{Da}	11.36 ^{Aa}	11.34 ^{Aa}	9.81 ^{Bde}	8.12 ^{Cefg}	8.10 ^{Cbdef}	
	5	7.15 ^{Ca}	8.72 ^{Bc}	11.14 ^{Aa}	11.14 ^{Aabc}	10.45 ^{Aa}	9.06 ^{Babcd}	
	7	7.17 ^{Aa}	6.65 ^{Ade}	6.31 ^{ABc}	5.56 ^{BCi}	5.18 ^{Ch}	4.73 ^{Ch}	
Mild heat	0	6.82 ^{Da}	11.49 ^{Aa}	11.13 ^{Aa}	10.19 ^{Bbcde}	9.04 ^{Cbcde}	8.74 ^{Cabcde}	
	1	6.82 ^{Da}	11.54 ^{Aa}	11.05 ^{Aa}	9.55 ^{Bde}	8.71 ^{BCdefg}	8.62 ^{Cbcde}	
	3	6.83 ^{Da}	11.39 ^{Aa}	11.26 ^{Aa}	9.53 ^{Bde}	7.96 ^{Cfg}	8.04 ^{Ccdefg}	
	5	6.81 ^{Da}	8.41 ^{Cc}	11.11 ^{ABa}	11.47 ^{Aa}	10.38 ^{Ba}	8.1 ^{Cbdef}	
	7	6.82 ^{Ca}	5.60 ^{De}	6.49 ^{CDc}	7.09 ^{BCh}	7.83 ^{Bg}	8.6 ^{Abced}	
Hydrogen Peroxide	0	7.25 ^{Da}	11.39 ^{Aa}	11.31 ^{ABa}	10.43 ^{Babcd}	9.31 ^{Cbcd}	9.18 ^{Cabc}	
	1	7.33 ^{Da}	11.42 ^{Aa}	11.18 ^{Aa}	10.23 ^{Bbcde}	8.95 ^{Cbdef}	8.74 ^{Cabcde}	
	3	7.33 ^{Da}	10.53 ^{Aab}	11.43 ^{Aa}	10.46 ^{Babcd}	8.45 ^{Cdefg}	7.88 ^{CDdefg}	
	5	7.30 ^{Ca}	8.17 ^{BCc}	8.61 ^{Bb}	9.70 ^{Ade}	9.93 ^{Aabc}	9.77 ^{Aa}	
	7	7.33 ^{Aa}	7.08 ^{ABd}	6.39 ^{Bc}	5.20 ^{Ci}	4.09 ^{Di}	3.14 ^{Ei}	
UV light	0	7.19 ^{Ca}	11.41 ^{Aa}	11.09 ^{Aa}	9.53 ^{Bde}	8.99 ^{Bcdef}	8.79 ^{Babcde}	
	1	7.24 ^{Ca}	11.48 ^{Aa}	10.99 ^{Aa}	9.35 ^{Bef}	8.8 ^{Bdefg}	8.63 ^{Dbcde}	
	3	7.20 ^{Da}	11.42 ^{Aa}	11.16 ^{Aa}	9.56 ^{Bde}	8.32 ^{Cdfeg}	7.77 ^{CDefg}	
	5	7.19 ^{Ca}	10.20 ^{Bb}	11.21 ^{Aa}	11.17 ^{Aab}	9.83 ^{Babc}	7.42 ^{Cfg}	
	7	7.15 ^{Ba}	7.14 ^{Bd}	7.38 ^{Bc}	8.05 ^{ABgh}	8.62 ^{Adefg}	8.86 ^{Aabcd}	

Table 1 (continued)

^{ABC}LS Means with different capital letters within a row are significantly different ($P < 0.05$).

^{abc}LS Means with different lowercase letters within a column to include all treatments are significantly different ($P < 0.05$).

Table 2. Probability > F Value ($P > F$) for fixed effects of the salt tolerance of *Lactococcus lactis* R-604 influenced by previous exposure to ethanol, hydrogen peroxide, mild heat, or UV light (Group 1).

Effect	P> F Group 1
Stress conditions	0.1661
Salt Concentration	<0.0001
Stress conditions*Salt concentrations	<0.0001
Days	<0.0001
Stress conditions*Days	0.0174
Salt concentrations*Days	<0.0001
Stress conditions*Salt concentrations*Days	<0.0001

On the fourth day, decline phase continued for 0, 1, 3 and 5% w/v NaCl and bacteria subjected to 7% w/v NaCl reached counts of 9 log CFU/ mL. On the fifth day, bacteria subjected to 0 and 7% w/v NaCl had significantly ($P < 0.05$) higher counts (about 2 logs) than the bacteria exposed to 5% w/v NaCl. Salt concentrations of 0, 1, 3 and 7% w/v had no significant ($P < 0.05$) differences in counts between them on day 5.

Uguen et al. (1999) reported significant reduction in growth (70%) of *Lactococcus lactis* ADRIA 85LO30 at 0.4 M NaCl and inhibition of growth was produced at 0.5 M NaCl or higher which is equivalent to 3% w/v NaCl. Obis et al. (2001) while studying the betaine transport system reported that several strains of *L. lactis* subsp. *lactis* and *cremoris* can grow at 6.5% w/v NaCl, but for some others strains growth was negatively affected at 2% w/v NaCl and above. Both studies showed that growth of *L. lactis* is inversely affected by the increase of osmolality (salt concentration in the solution) but they did not test any treatment or stress condition prior the osmotic shock. Conversely, results obtained in the present study showed normal growth of *L. lactis* R-604 at up to 5% w/v NaCl compared to 0% w/v NaCl, this difference in results could be

caused by the different strains used in each study. Levels of salt from 0 to 5% w/v showed a similar growth during the 5 days. However, decline of growth was observed for three days at 7% w/v NaCl.

Kasımoğlu et al. (2004) used a *Lactococcus lactis* R-707 in a 90 days study in white cheese, and they found that major changes occurred during the first 5 days, the culture grew from 7 to 10 log CFU/ mL and on day 5 counts slowly decreased and bacteria reached stationary phase thereafter. Vinderola et al. (2003) showed the influence of different compounds associated with fermented dairy products on the growth of lactic acid bacteria. The study includes NaCl and KCl at 1 and 2% w/v concentration and found no significant ($P>0.05$) difference in growth compared to control (0% w/v NaCl) for several commercial cultures of *L. lactis*. Other lactic acid bacteria such as *Lactobacillus* strains showed the same behavior under osmotic conditions, tolerating salt concentrations at 5% w/v and failing to grow at 7% NaCl (Sheehan et al., 2006).

3.3 Salt tolerance of *Lactococcus lactis* R-604 after ethanol stress (10% v/v for 30 minutes)

Counts of *Lactococcus lactis* R-604 salt tolerance influenced by ethanol exposure are presented in Table 1. On day 0, there were no significant ($P>0.05$) differences in counts at all salt concentrations, which mean that all salt concentrations started at the same point (approximately 7 log CFU's/mL). On day 1, *L. lactis* R-604 cells exposed to 0, 1 and 3% w/v NaCl grew 4 logs, and were significantly ($P<0.05$) higher than the bacterial cells subjected to 5 and 7% w/v NaCl. Bacterial cells subjected to ethanol at 5% w/v NaCl only grew 2 logs, and were significantly ($P<0.05$) lower than the non-ethanol treated bacteria (Control 1) at 5% w/v NaCl on day 1 (Table 3). Bacteria subjected to 5% w/v NaCl had significantly ($P<0.05$) higher counts than the bacteria exposed to 7% w/v NaCl which did not show any growth, similar to Control 1 at 7% w/v NaCl on day 1 (Table 3).

On day 2, bacterial cells subjected to 7% NaCl had significantly ($P<0.05$) lower counts than 0, 1, 3 and 5% w/v NaCl by approximately 5 logs (Table 1) but did not have any significant ($P>0.05$) difference compared to Control 1 at 7% w/v NaCl (Table 3). On day 3, bacterial cells subjected to 0% and 5% w/v NaCl had significantly ($P<0.05$) higher counts compared to 0, 1 and 7% w/v NaCl. However, bacteria exposed to ethanol at 5% NaCl had significantly ($P<0.05$) higher counts than the Control 1 at 5% NaCl. Bacterial cells subjected to the 7% w/v NaCl had the lowest counts on day 3 and were significantly ($P<0.05$) lower than Control 1 at the same salt concentration of 7% w/v (Table 3).

On the fourth day, bacteria subjected to 5% w/v NaCl had significantly ($P<0.05$) higher counts than 0, 1, 3 and 7% w/v NaCl and also were significantly higher than the Control 1 at 5% w/v NaCl (Table 1). Bacteria subjected to 7% w/v NaCl had the lowest counts of the ethanol treated cells and counts were significantly ($P<0.05$) lower than the Control 1 (Table 3). On the fifth day, there were no significant ($P>0.05$) differences between bacterial counts at 0, 1, 3 and 5% w/v NaCl, but ethanol treated bacterial cells at 5% w/v NaCl were significantly ($P<0.05$) higher than the Control 1 at 5% w/v NaCl. Growth of bacterial cells exposed to 7% w/v NaCl declined over the 5 days and showed significantly ($P<0.05$) lower counts than the Control 1 at 7% w/v NaCl (Table 3). Foucaud-Scheunemann and Poquet (2003) found that incremental addition of ethanol (up to 10% v/v) or NaCl (5% w/v) negatively affected *L. lactis* growth by reducing approximately 1 log CFU/mL in only 4 hours of exposure, but they did not evaluate the salt tolerance after ethanol exposure.

Table 3. Probability values (P values) for the paired comparison between the ethanol treated *Lactococcus lactis* R-604 cells and the Control 1 (no previous treatment).

Salt Concentration (w/v %)	Time (Days)					
	0	1	2	3	4	5
0	0.9707	0.7838	0.7525	0.4132	0.4475	0.0548
1	0.9022	0.8409	0.8859	0.8119	0.6845	0.4580
3	0.9858	0.9411	0.6126	0.9382	0.8337	0.9080
5	0.9171	0.0015	0.9779	0.0584	0.0022	0.0002
7	0.9162	0.7745	0.1291	<.0001	<.0001	<.0001

According to van Bokhorst-van de Veen et al. (2011) ethanol pre-exposure can only confer cross protection effect against heat stress since the damage that ethanol produces to cell membrane affects mainly its permeability, fluidity and also destabilizes enzymes on the membrane but increases the transcription of heat stress associated proteins. Reproductive ability and growth of bacterial cell are affected by increasing concentrations of ethanol in medium and defense mechanisms varies among species but mainly affect cell membrane fatty acid composition which is also damaged under high osmotic conditions (Taylor et al., 2008). However, Sharma (1997) reported that prior exposure to NaCl (3 to 13% w/v) increase tolerance to ethanol (16% v/v) in *Sacharomyces cerevisiae* due to an increase of trehalose in the cell membrane but salt tolerance after ethanol exposure was not evaluated.

3.4 Salt tolerance of *Lactococcus lactis* R-604 after mild heat (52° C for 30 minutes)

Data for the salt tolerance of mild heat treated samples of *L. lactis* R-604 is presented in Table 1. On day 0, all samples had statistically the same ($P > 0.05$) bacterial counts at approximately 7 log CFU's/ mL, meaning that all samples had the same starting point for all salt concentrations. After the first day of incubation, *L. lactis* R-604 cells subjected to 0, 1 and 3% w/v NaCl showed an equal ($P > 0.05$) exponential growth of 4 logs, bacteria subjected to 5% w/v NaCl only grew 1 log and bacteria subjected to 7% w/v were reduced by 1 log. Mild heat treated bacterial cells at 5

and 7% w/v NaCl had significantly ($P<0.05$) lower bacterial counts than the non-heated bacterial cells (Control 1) on day 1 (Table 4). On the second day, cells subjected to 0, 1, 3 and 5% w/v NaCl had no significant ($P> 0.05$) difference in bacterial counts between them and stayed at 11 log CFU/ mL while cells subjected to 7% were significantly ($P<0.05$) lower (Table 1).

On the third day, cells subjected to mild heat at 5% w/v NaCl showed significantly ($P<0.05$) higher bacterial counts than 0, 1, 3, 7% w/v NaCl and the Control 1 at 5% w/v NaCl (Table 4). Bacterial cells subjected to 7% w/v NaCl had the lowest ($P<0.05$) bacterial counts of the mild heated cells and were also had significantly ($P<0.05$) lower bacterial counts than the Control 1 at 7% w/v NaCl (Table 4). On the fourth day, 5% w/v NaCl had significantly ($P<0.05$) higher bacterial counts than the mild heated cells at 0, 1, 3, 7% w/v NaCl and the Control 1 at 5% w/v (Table 4). Bacterial cells subjected to 0, 1, 3 and 7 had no significant ($P > 0.05$) differences in counts between them on day 4 (Table 1). Mild heat treated cells at 7% w/v NaCl had significantly ($P<0.05$) lower bacterial counts than the Control 1 at 7% w/v NaCl (Table 4). On the fifth day, there were no significant ($P> 0.05$) difference in bacterial counts among the mild heat treated samples at all different salt concentrations but bacterial counts of the heat treated cells at 5% w/v were significantly ($P<0.05$) higher than the Control 1 at 5% w/v (Table 4).

Smith et al. (2010) stated that *L. lactis* had better growth and acid production at salt concentrations between 3 and 5% w/v NaCl. Exposure to heat (39°C) can increase resistance to NaCl, since there is evidence that heat stress induced proteins like htrA are also used under osmotic stress as a defense mechanisms (Foucaud-Scheunemann and Poquet, 2003). Kilstrup et al. (1997) showed that after 4 hours of salt exposure (2.5% w/v NaCl concentrations and higher) *L. lactis* growth was negatively affected. This study showed that several heat stress genes such as

GroES, GroEl, DnaK can also be produced under osmotic stress but the influence of heat on the salt tolerance of *L. lactis* was not studied.

Heat induced proteins have also been associated with osmoregulation in Gram-negative bacteria that protects the cell from dehydration (Forns et al., 2005). This gene production overlap can explain the slight increase in salt tolerance of bacteria subjected to 5% w/v NaCl after hydrogen peroxide and heat stress since different stress conditions can make the cell produce the same type of membrane associated proteins in order to protect the cell.

Table 4. Probability values (P values) for the paired comparison between mild heated *Lactococcus lactis* R-604 cells and the Control 1 (no previous treatment).

Salt Concentration (w/v %)	Time (Days)					
	0	1	2	3	4	5
0	0.5303	0.9571	0.8972	0.8256	0.8050	0.3812
1	0.5650	0.9248	0.9366	0.5244	0.4849	0.7649
3	0.5715	0.9897	0.7123	0.6654	0.6081	0.9949
5	0.5988	0.0002	0.9237	0.0126	0.0034	0.0482
7	0.5994	0.0257	0.2334	0.0188	0.0092	0.5462

Desmond et al. (2002) showed that heat resistance can be achieved by previous sub-lethal exposure to salt (approximately 2% w/v NaCl) because some of the genes produced under both stresses are the same. However, a study from Smith et al. (2012), showed that heat adapted (37.5°C) strains of *L. lactis* can develop an osmotic hypersensitivity (at around 0.25 M NaCl).

3.5 Salt tolerance of *Lactococcus lactis* R-604 after oxidative stress (15 mM of hydrogen peroxide for 30 minutes).

Counts of salt tolerance of *Lactococcus lactis* R-604 after oxidative stress at various salt concentrations for 5 days are presented in Table 1. On day 0, all samples treated with hydrogen peroxide started with statistically the same ($P>0.05$) counts at about 7 log CFU/mL. On day 1, *L.*

lactis R-604 cells subjected to 0, 1 and 3% w/v NaCl had significantly ($P<0.05$) higher than 5 w/v NaCl by more than 2 logs and 4 logs more than bacteria subjected to 7% w/v NaCl. On the second day, bacterial cells subjected to 0, 1 and 3% had no significant ($P>0.05$) difference in counts while the cells subjected to 5 and 7% w/v NaCl showed the lowest ($P<0.05$) bacterial counts. Bacterial cells subjected to 5% NaCl w/v had significantly ($P<0.05$) lower counts (by 2 logs) than the non-hydrogen peroxide treated cells (Control 1) on days 1 and 2 (Table 5).

On the third day, there were no significant ($P<0.05$) differences in counts between bacterial cells subjected to 0, 1, 3 and 5% w/v NaCl, while the 7% w/v NaCl had lowest ($P<0.05$) counts. Cells subjected to hydrogen peroxide at 7% w/v had significantly ($P<0.05$) lower bacterial counts than the Control 1 at 7% w/v NaCl on day 3. On the fourth day, bacteria subjected to 5% w/v NaCl had significantly ($P<0.05$) higher counts than the 3 and 7% w/v NaCl by 1 and 5 log respectively and were also higher than the Control 1 at 5% w/v NaCl. Bacteria subjected to 0, 1 and 3% w/v NaCl had no significant ($P>0.05$) differences in counts between them. This tendency continued until day 5. Prior exposure to hydrogen peroxide at 5% w/v NaCl showed an enhancement in salt tolerance on days 4 and 5. Conversely, bacterial counts at 7% w/v NaCl were reduced over the 5 days and were significantly ($P<0.05$) lower than Control 1 (by up to 5 logs) on days 3, 4 and 5 (Table 5).

The 30 minutes of 15 mM hydrogen peroxide exposure in this study was not enough to reduce bacterial counts of *L. lactis* R-604 on day 0, but it weakened the bacterial cells against hyperosmotic conditions (7% w/v NaCl). It took 3 days for the cell to adapt to the 5% w/v NaCl and enhanced its resistance to salt over time. However, hydrogen peroxide stress had a negative effect on the salt tolerance of *L. lactis* at 7% w/v NaCl since it drastically reduced its counts from day 3.

Table 5. Probability values (P values) for the paired comparison between the hydrogen peroxide treated *Lactococcus lactis* R-604 cells and the Control 1 (no previous treatment).

Salt Concentration (w/v %)	Time (Days)					
	0	1	2	3	4	5
0	0.8869	0.9107	0.6561	0.5065	0.4531	0.9955
1	0.7090	0.9131	0.7585	0.5476	0.7930	0.6046
3	0.7297	0.1141	0.5006	0.2012	0.7041	0.7765
5	0.7186	<.0001	<.0001	0.4662	0.0324	<0.001
7	0.6900	0.6381	0.1689	<.0001	<.0001	<.0001

Lactococcus's lack of catalase activity made it vulnerable for oxidative stress which often leads to DNA and cell membrane damage that may have weakened the bacterial cells and prevented them from growing at 7% w/v NaCl (Rochat et al., 2005). According to Miyoshi et al. (2003) 0.2 mM of hydrogen peroxide is enough to inhibit 50% the growth capacity of *L. lactis* and the damage produced to the fatty acids at cell membrane may also affect its permeability and osmoregulation. Exposure of *L. lactis* to hydrogen peroxide at only 4 mM can reduce 4 log CFU's/mL in one hour (Rochat et al., 2005).

3.6 Salt tolerance of *Lactococcus lactis* R-604 after UV light exposure (254 nm for 5 min)

Counts of salt tolerance of *Lactococcus lactis* R-604 after UV light exposure is presented in Table 1. No significant ($P > 0.05$) differences were found at the initial counts (Day 0) between all salt concentrations, which meant that all samples had the same starting point. On day 1, *L. lactis* R-604 cells subjected to 0, 1 and 3% w/v NaCl grew more than 4 logs and had significantly ($P < 0.05$) higher bacterial counts than the cells subjected to 5 and 7% w/v NaCl. Bacteria subjected to 5% w/v NaCl grew 3 logs and had significantly ($P < 0.05$) higher counts than the 7% w/v NaCl which did not show any growth. On the day 2, there were no significant ($P > 0.05$) differences in bacterial counts between 0, 1, 3 and 5% w/v NaCl. Bacteria subjected to 7% w/v

NaCl started to grow on day 2 but counts remained significantly ($P<0.05$) lower than 0, 1, 3 and 5% w/v NaCl.

On the third day, bacterial cells subjected to 5% w/v NaCl had the highest ($P<0.05$) counts while cells subjected to 0, 1 and 3% w/v NaCl had significantly ($P<0.05$) lower bacterial counts by 2 logs and did not have significant ($P>0.05$) differences between them. Bacteria subjected to 7% w/v had the lowest ($P<0.05$) counts. On day 4, bacteria subjected to 0, 1 and 5% w/v showed no significant ($P>0.05$) difference in counts between them and were significantly ($P<0.05$) higher than the counts obtained by bacteria subjected to 3 and 7% w/v NaCl (Table 1).

On the fifth day, bacteria subjected to 0, 1 and 7% w/v NaCl showed no significant ($P>0.05$) difference in counts between them and were the highest of all samples treated with UV light. There were not any significant ($P>0.05$) differences in counts between the UV light treated bacteria and the non-treated (Control 1) on any moment at any salt concentration showing no improvement to the salt tolerance (Table 6). The present study avoided excessive UV light exposure because bacteria are very sensitive to UV light and long exposure may produce severe DNA damage which could have killed the bacterial cells (Pfeifer et al., 2005).

Table 6. Probability values (P values) for the paired comparison between the UV light treated *Lactococcus lactis* R-604 cells and the Control 1 (no previous treatment).

Salt Concentration (w/v %)	Time (Days)					
	0	1	2	3	4	5
0	0.9710	0.9298	0.9562	0.3262	0.8715	0.3350
1	0.8377	0.9929	0.9752	0.3097	0.6025	0.7492
3	0.9069	0.9739	0.8630	0.7047	0.8785	0.6271
5	0.8621	0.6117	0.9295	0.0518	0.0507	0.4599
7	0.9472	0.5738	0.6624	0.5397	0.2410	0.9101

Eichenbaum and Livneh (1998) showed evidence that *E. coli* can mutate after UV light exposure and can help to increase resistance to other environmental stresses. Hartke et al. (1995) showed

that *L. lactis* exposed to 100J/cm² can develop resistance to ethanol (20% v/v) and acid stress (pH 4.0) due to an overlap in stress protein expression. In other study of Hartke et al. (1997) 30 minutes of UV light exposure at 254 nm were necessary to start the production of heat stress proteins such as GroEL and GroES which can also lead to a cross protection effect.

3.7 Overall comparison between ethanol, hydrogen peroxide, mild heat, UV light with Control 1 (no previous stress).

A comparison between all stress conditions in Group 1 is presented in Table 1. There were no significant ($P>0.05$) differences in the initial counts (Day 0) between all stress conditions at all salt concentrations. On the first day, no significant ($P>0.05$) differences were found between all treatments grown at 0, 1 and 3% w/v NaCl. Regarding 5% w/v NaCl the non-treated (Control 1) and UV light exposed bacteria had the highest ($P<0.05$) counts. Ethanol or mild heat at 7% w/v NaCl showed the lowest counts of all stress conditions on day 1.

On the second day, most of the treatments reached their highest ($P<0.05$) counts. There were no significant ($P>0.05$) differences between bacterial cells subjected to 0, 1, and 3% w/v after ethanol, mild heat, UV light and the non-treated cells (Control 1). Bacterial cells treated with ethanol, heat shock and hydrogen peroxide at 5% w/v NaCl had significantly ($P<0.05$) lower counts compared to Control 1 and UV light. Bacteria subjected to ethanol and hydrogen peroxide at 7% w/v NaCl obtained the lowest ($P<0.05$) counts on day 2. Ethanol and oxidative stress are very damaging environmental conditions for *L. lactis*. These stresses can disrupt metabolic pathways and have a bacteriostatic and bactericidal effects (Miyoshi et al., 2003, Liu and Qureshi, 2009). Exposing bacteria to ethanol, heat shock and oxidative stress and then to a high osmotic condition (7% w/v NaCl) limited their growth.

On the third day, no effect of stress conditions were found in cells subjected to 0, 1 and 3% w/v NaCl. Salt tolerance of *L. lactis* was enhanced after ethanol or heat shock exposure at 5% w/v NaCl since bacterial counts were significantly ($P<0.05$) higher compared to Control 1. *L. lactis* R-604 cells subjected to all stress conditions at 7% w/ NaCl showed the lowest ($P<0.05$) counts. On day 4 and 5, there was a large overlap in bacterial counts mean's significant ($P<0.05$) differences (Table 1). On day 4, the highest ($P<0.05$) means were obtained by ethanol or heat treated bacterial cells subjected to 5% w/v NaCl. Conversely, bacteria treated with ethanol or hydrogen peroxide and then subjected to 7% w/v NaCl had the lowest ($P<0.05$) counts. On day, 5 highest ($P<0.05$) counts were found for hydrogen peroxide treated cells subjected to 5% w/v NaCl while the lowest ($P<0.05$) were found on ethanol or hydrogen peroxide subjected to 7% w/v NaCl.

The major effect of osmotic stress by sodium chloride in *L. lactis* is dehydration (Sanders et al., 1999). The proposed defense mechanisms are osmolyte transport system (which include proline and glycine betaine accumulation in cytoplasm to counteract the osmotic imbalance), and also the gene response of the cell by the expression of stress induced proteins such as dnak, groELS, dnaJ, DnaK, and RecA, which are also induced by heat stress (Sanders et al., 1999). Kim et al. (2002) explained that bacterial cells subjected to two consecutive stresses may fail to develop resistance since in order to increase stress resistance cells must be subjected to a recovering period after the first stress, which allow them to synthesize the proteins needed for survival of further lethal challenges.

3.8 Influence of osmotic adaptation and lactose deprivation for 24 hours on the salt tolerance of *Lactococcus lactis* R-604.

Counts of salt tolerance of *Lactococcus lactis* R-604 at after osmotic adaptation (3% w/v NaCl) or lactose deprivation for 24 hours are presented in Table 7. For Group 2, the three way interaction had no significant ($P>0.05$) effect, but the two way interaction (Salt Concentration*Days) showed a significant ($P<0.05$) effect which meant that cell counts only depended on the salt concentration and days of exposure (Table 8).

Salt tolerance of *Lactococcus lactis* R-604 as influenced by 24 hours incubation period (Control 2).

Growth of *Lactococcus lactis* R-604 at various salt concentrations after 24 hours incubation period under ideal conditions (M17 broth supplemented with 0.5% w/v lactose at 30°C) is presented in

Table 7. Least Square Means (LS Means) expressed as log CFU/ml of salt tolerance of *Lactococcus lactis* R-604 after 24 hours exposure to 3% NaCl (osmotic adaptation) or lactose deprivation.

			Days							
			0	1	2	3	4	5	6	
Control 2	Salt (% w/v NaCl)	0	7.16 ^{Ea}	9.81 ^{BCa}	11.27 ^{Aa}	10.53 ^{ABabc}	9.14 ^{CDbc}	8.71 ^{Dab}	8.43 ^{Dabc}	
		1	7.16 ^{Ca}	9.32 ^{Ba}	11.32 ^{Aa}	10.63 ^{Aab}	9.12 ^{BCbc}	8.42 ^{Cab}	8.62 ^{BCabc}	
		3	7.16 ^{Da}	9.31 ^{Ba}	11.28 ^{Aa}	9.77 ^{Bbcd}	8.49 ^{Cc}	7.78 ^{CDb}	7.78 ^{CDcd}	
		5	7.16 ^{CDa}	9.43 ^{Ba}	11.15 ^{Aa}	11.04 ^{Aa}	9.76 ^{Bab}	7.72 ^{Cb}	6.82 ^{De}	
		7	7.16 ^{Ba}	9.38 ^{Aa}	8.72 ^{Ab}	8.95 ^{Ade}	9.01 ^{Abc}	8.88 ^{Aab}	8.88 ^{Aab}	
Osmotic adaptation	Salt (% w/v NaCl)	0	7.02 ^{Ea}	10.0 ^{Ba}	11.23 ^{Aa}	9.66 ^{BCcd}	9.04 ^{CDbc}	8.66 ^{Dab}	7.97 ^{Dbcd}	
		1	7.02 ^{Da}	10.0 ^{Ba}	11.18 ^{Aa}	10.36 ^{ABabc}	8.98 ^{Cbc}	8.77 ^{Cab}	8.14 ^{Cbcd}	
		3	7.02 ^{Fa}	9.96 ^{Ba}	11.25 ^{Aa}	10.42 ^{ABabc}	8.63 ^{CDc}	7.96 ^{DEb}	7.29 ^{EFde}	
		5	7.03 ^{CDa}	9.84 ^{Ba}	11.2 ^{Aa}	10.98 ^{Aa}	10.08 ^{Ba}	7.62 ^{Cb}	6.76 ^{De}	
		7	7.03 ^{Ca}	9.64 ^{Aa}	9.01 ^{Ab}	9.41 ^{Acde}	9.38 ^{Aabc}	9.25 ^{ABa}	8.5 ^{Babc}	
Lactose deprivation	Salt (% w/v NaCl)	0	7.10 ^{Da}	9.49 ^{Ba}	11.31 ^{Aa}	10.89 ^{Aa}	9.34 ^{BCabc}	8.83 ^{BCab}	8.65 ^{Cabc}	
		1	7.10 ^{Ca}	9.42 ^{Ba}	11.32 ^{Aa}	10.67 ^{Aab}	8.98 ^{Bbc}	8.76 ^{Bab}	8.73 ^{Bab}	
		3	7.10 ^{Da}	9.45 ^{Ba}	11.34 ^{Aa}	10.73 ^{Aa}	8.66 ^{BCc}	8.11 ^{Cb}	8.16 ^{Cbcd}	
		5	7.10 ^{Da}	9.44 ^{Ba}	11.18 ^{Aa}	11.06 ^{Aa}	10.11 ^{Ba}	8.03 ^{Cb}	6.65 ^{De}	
		7	7.10 ^{Ca}	9.38 ^{Aa}	8.21 ^{Bb}	8.53 ^{Be}	8.58 ^{ABc}	9.15 ^{Aa}	9.23 ^{Aa}	

Table 7 (continued)

^{ABC}LS Means with different capital letters within a column are significantly different ($P<0.05$).

^{abc}LS Means with different lowercase letters within a row to include all treatments are significantly different ($P<0.05$).

Table 8. Probability > F Value ($P > F$) for fixed effects of the salt tolerance of *Lactococcus lactis* R-604 influenced by 24 hours osmotic adaptation or lactose deprivation (Group 2).

Effect	P> F Group 2
Stress conditions	0.9002
Salt Concentration	0.0010
Stress conditions*Salt concentrations	0.6911
Days	<0.0001
Stress conditions*Days	0.0823
Salt concentrations*Days	<0.0001
Stress conditions*Salt concentrations*Days	0.9984

Table 7. On day 0, there were no significant ($P>0.05$) difference in counts between all salt concentrations after 24 hours of incubation. On day 1, all salt concentration showed an exponential growth of more than 2 logs with no significant ($P>0.05$) differences. On day 2, bacterial cells subjected to 7% w/v NaCl decreased 1 log, while the cells subjected to 0, 1, 3 and 5% w/v NaCl grew 2 logs more, making a significant ($P<0.05$) difference of 3 logs between them.

On the third day, *L. lactis* R-604 cells subjected to 5% w/v NaCl showed the highest ($P<0.05$) counts. Bacteria subjected to 0, 1 and 3% w/v NaCl showed no significant ($P>0.05$) difference in counts between them and cells subjected to 7% w/v NaCl presented the lowest ($P<0.05$) counts. On the fourth day, bacterial cells subjected 5% w/v NaCl were significantly ($P<0.05$) higher than those subjected to 3% NaCl. On day 5, there were no significant ($P>0.05$) differences in counts between all levels of salt. Conversely, on day 6 bacteria subjected to 7% w/v NaCl showed significantly ($P<0.05$) higher counts than 3 and 5% w/v NaCl. Bacteria subjected to 0, 1 and 3%

w/v NaCl had no significant ($P>0.05$) difference in counts between them at this time whereas bacteria subjected to 5% w/v were the lowest ($P<0.05$).

The growth inhibition produced by the sudden osmotic stress could cause the loss of cell turgor pressure and therefore its dehydration. Thus affecting the bacteria's primary functions and reducing the reproduction rate (Booth, 1998). The accumulation of compatible solutes has been proposed as a mechanism of overcome hyperosmotic conditions for many microorganisms (Gutierrez et al., 1995) and particularly glycine betaine and proline for lactic acid bacteria (Molenaar et al., 1993, Le Marrec, 2011). Osmotic lethal challenge (20 w/v NaCl in medium) leads to an activation of BusA operations to improve betaine uptake which can increase salt tolerance of *L. lactis* subsp. *lactis* but it was not found to increase salt tolerance of subsp. *cremoris* (Obis et al., 2001).

3.9 Salt tolerance of *Lactococcus lactis* R-604 as influenced by 24 hours osmotic adaptation.

Counts of *Lactococcus lactis* R-604 after 24 hours of osmotic adaption are presented in Table 7. On day 0, there were no significant ($P>0.05$) differences in counts for the osmotic adaptation period of 24 hours. On day 1, *L. lactis* R-604 cells grew 3 logs and there were no significant ($P>0.05$) differences in bacterial counts between all salt concentrations. On the second day, bacteria subjected to 7% w/v decreased in counts while all others (0, 1, 3 and 5% w/v NaCl) grew at 11 log CFU's/ mL. On day 3, osmotic adapted bacterial cells subjected to 5% w/v NaCl had the highest ($P<0.05$) counts; 0, 1 3 and 7% w/v NaCl had no significant ($P>0.05$) differences in counts between them (Table 7). On the fourth day, bacteria subjected to 5% w/v NaCl had significantly ($P<0.05$) higher counts than 0, 1 and 3% w/v NaCl by 1 log. On day 5, bacteria subjected to 7% w/v NaCl had the highest counts and there were no significant ($P<0.05$)

differences between 0 and 1% w/v NaCl. On the sixth day, bacteria subjected to 3 and 5% had significantly ($P<0.05$) lower counts than 0, 1 and 7% which did not have significant ($P>0.05$) differences between them.

Table 9. Probability values (P values) for the paired comparison between the osmotic adapted *Lactococcus lactis* R-604 cells and the Control 2 (24 hours incubation under ideal conditions).

Salt Concentration (w/v %)	Time (Days)						
	0	1	2	3	4	5	6
0	0.7638	0.6802	0.9167	0.0637	0.8172	0.9079	0.3227
1	0.7638	0.1439	0.7705	0.5650	0.7580	0.4495	0.3104
3	0.7638	0.1608	0.9512	0.1620	0.7711	0.1771	0.3025
5	0.7683	0.3699	0.9224	0.8951	0.4861	0.8366	0.8891
7	0.7683	0.5787	0.5401	0.3291	0.4267	0.4160	0.4191

There was no adaption effect after exposing the *L. lactis* R-604 cells to mild salt concentration (3% w/v NaCl) and then transfer them to different salt concentrations both lower or higher (0, 1, 3, 5 and 7% w/v NaCl) since no significant ($P>0.05$) differences were found on the paired comparison between the osmotic adapted *L. lactis* R-604 cells and Control 2 (Table 9). The 24 hours osmotic adaptation at 3% w/v NaCl did not have a significant ($P>0.05$) effect on the salt tolerance of *Lactococcus lactis* R-604.

Results defer from previous studies of adaptation in different microorganisms which have shown to enhance tolerance to acid and heat stress after a mild stress exposure (Leyer et al., 1995, Gahan et al., 1996, Davidson and Harrison, 2002, Desmond et al., 2002, van Bokhorst-van de Veen et al., 2011). Smith et al. (2012) showed that heat resistance strains of *L. lactis* can be develop by a mild heat shock (34° C) for 3 hours and a recovering time. Acid tolerance at pH 3.9 has been improved by prior exposure of different strains of *L. lactis* strains to pH 5.5. A study from Tripathi et al. (2003) showed that pre-exposure of *L. lactis* to 3% w/v NaCl in Elikér's

broth or in milk can enhance its growth and acid production when it is transferred to up to 3% NaCl w/v but they failed to increase its growth or acid production at higher salt concentrations.

3.10 Salt tolerance of *Lactococcus lactis* R-604 as influenced by 24 hours lactose deprivation.

Data of salt tolerance of *Lactococcus lactis* R-604 after lactose deprivation is presented in Table 7. On day 0, all salt concentrations had statistically the same ($P>0.05$) counts at approximately 7 log CFU/ mL. After the first 24 hours of incubation *L. lactis* R-604 cells grew 2 logs with no significant ($P>0.05$) difference in counts between them and in the same fashion than the 24 hours incubated under ideal conditions (Control 2) and osmotic adapted cells. On days 2 and 3, there were no significant ($P>0.05$) differences in counts between bacteria subjected to 0, 1, 3 and 5% w/v NaCl while bacterial counts at 7% w/v NaCl were significantly ($P<0.05$) lower. On day 4, lactose deprived *L. lactis* R-604 cells at 0 and 5% w/v NaCl had the highest ($P<0.05$) bacterial counts while cells subjected to 7% w/v NaCl had the lowest ($P<0.05$). Conversely, on day 5 the highest ($P<0.05$) counts were obtained by the bacterial cells subjected to 7% w/v NaCl and for 0, 1, 3 and 5% w/v NaCl and there were no significant ($P>0.05$) differences in counts between them. On the sixth day, cells subjected to 0, 1 and 7% w/v NaCl obtained the highest counts, while 5% w/v NaCl were the lowest ($P<0.05$). Cells under 5% w/v showed a drastic decrease in counts from day 4 (10 log CFU/ mL) to day 6 (6.6 log CFU/ mL).

Interestingly, lactose deprived *Lactococcus lactis* R-604 cells grew at the same rate of Control 2 regardless of the salt concentration. The only significant ($P<0.05$) difference found was on day 3, at 3% w/v NaCl, where the lactose deprived cells showed 1 log higher counts than the cells grown 24 hours under ideal conditions (Control 2), at the same salt concentration on the same day (Table 10). Hartke et al. (1994) showed that resistance to heat, ethanol, low acid, hydrogen

peroxide and NaCl (3.5% w/v) can be achieved by stationary phase cells of *L. lactis* grown under lactose starvation. Other microorganisms such as *Enterococcus faecalis* and *Listeria monocitogenes*, after complete glucose starvation, can develop resistance to heat, hydrogen peroxide, bile salt and sodium chloride (Giard et al., 1996, Lou and Yousef, 1996). Jenkins et al. (1990) showed that *E. coli* can develop resistance to osmotic shock (2.5% w/v NaCl) after 4 hours of glucose starvation.

Table 10. Probability values (P values) for the paired comparison between lactose deprived *Lactococcus lactis* R-604 cells and the Control 2 (24 hours incubation under ideal conditions).

Salt Concentration (w/v %)	Time (Days)						
	0	1	2	3	4	5	6
0	0.8873	0.4916	0.9362	0.4328	0.6805	0.7898	0.6466
1	0.8873	0.8256	0.9871	0.9365	0.7663	0.4675	0.8152
3	0.8873	0.7648	0.9027	0.0407	0.7238	0.4710	0.4118
5	0.8873	0.9778	0.9585	0.9663	0.4392	0.4980	0.7106
7	0.8873	0.9982	0.2730	0.3676	0.3513	0.5486	0.4579

The main proposed explanation for the cross-protection that starvation stress can confer to bacteria is the pattern change in gene expression. This leads to different polypeptides synthesis, stress induced proteins, that are produced under more than one environmental stressor (Pichereau et al., 2000). However, a study involving glucose starved organisms failed to improve resistance to hydrogen peroxide stress, since the specific catalase enzymes (KatA and KatE) were lower than the catalase activity generated after an oxidative adaptation (Engelmann and Hecker, 1996).

3.11 Overall comparison of osmotic adaption, lactose deprivation with Control 2.

Only one significant ($P<0.05$) difference was found between the lactose deprived cells and the Control 2, at the 3% w/v NaCl on day 3 (Table 9). According to Kilstrup et al. (1997), cells usually reduce metabolic activity in order to start an adaptation process. Kim et al. (2002)

showed that cells in stationary phase are more resistant to stress than cells in exponential phase. Cells in Group 2 were in stationary phase at the time of transfer them to the different salt concentrations. Nevertheless, in this experiment *L. lactis* R-604 cells were not stressed enough to reduce their growth on day 1, which did not trigger their defense mechanisms. Kim et al. (2002) also stated that in order to generate an adaptation response cells must be subjected to a mild stress for an adequate amount of time thus allow them to synthesize numerous proteins to protect the cell structure against the stress applied.

Control 1 data shows that 7% w/v NaCl was necessary to prevent *L. lactis* from growth of (Table 1), thus for further studies this salt concentration should be considered to trigger an osmotic adaptation response. Regarding lactose deprivation, other ways to induce starvation stress include be by growing the cells in suspension media such as saline solution (0.08% w/v NaCl), peptone water or Phosphate Buffer Saline solution (PBS). However Hartke et al. (1994) stated that small amounts of carbohydrates (0.1% w/v glucose) in media can be used rapidly by cells and trigger stationary phase quicker and hence helping the cells adapt better to further stress conditions.

It must also be considered that during starvation treatments amino acids must be present in media as a precursor for protein synthesis to obtain better stress adaptation (van de Guchte et al., 2002). Prior exposure of *L. lactis* R-604 in 3% w/v NaCl or lactose starvation and subsequent use of treated cells in up to 5% w/v NaCl did not have a negative influence on its growth which could be industrially feasible for high salt (5% w/v NaCl) foods.

CHAPTER 4: CONCLUSIONS

Results obtained from this study showed that salt tolerance of *Lactococcus lactis* R-604 was enhanced after ethanol or mild heat exposure at 5% w/v NaCl on days 3, 4 and 5; after hydrogen peroxide exposure at 5% w/v NaCl on days 4 and 5; and after lactose starvation at 3% w/v NaCl on day 3. Growth of *L. lactis* R-604 was not negatively affected by any of the stress conditions applied at salt concentrations of 0, 1 and 3% w/v. The culture was able to grow in 3% w/v NaCl or no lactose after 24 hours and these stress conditions did not affect its salt tolerance. Growth was maintained at 7% w/v NaCl regardless the stress conditions. The combination of ethanol or hydrogen peroxide with a salt concentration of 7% w/v had a negative effect on the growth of *L. lactis* R-604.

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VITA

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